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Factors Influencing Reproduction and Genetic Toxic Effects on Male Gonads

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The objective of toxicological study of a target organ, such as the testis, is to elucidate the qualitative and quantitative toxic effects of a chemical on that organ. The ultimate objective is to assess the toxic effects of a chemical in laboratory animals and extrapolate the pertinent experimental data to man. To accomplish these objectives, one must consider the main factors which may influence and modulate the toxic effects of chemicals in the organ. In the male gonads, such modifying factors are the pharmacokinetic parameters governing the absorption, distribution, activation and detoxification of indirect carcinogens, covalent bindings to macromolecules, and DNA damage as well as DNA repair of damaged germ cells. All of these factors have been presently studied in our laboratory and are discussed in this paper with the exception of covalent bindings to macromolecules.

The pharmacokinetic studies demonstrated that the functional blood–testis barrier (BTB) closely resembles the blood-brain barrier in transport characteristics: the permeability of nonelectrolytes and the acidic drugs with pK_a values depend upon their molecular size and their partition coefficients, respectively. Thus, the functional BTB, restricts the permeability of many foreign compounds to male germ cells. Studies of mixed function oxidases and cytochrome P-450 system in male gonads demonstrated that the presence of AHH, EH, and GSH-ST implicate activation and detoxification of polycyclic hydrocarbons. Thus, active electrophiles may exert significant toxic effects locally within both interstitial and germ cell compartments. The presence of an efficient DNA repair system in premeiotic spermatogenic cells (and not in spermiogenic cells) can further modify both toxic and mutagenic events in the subsequent differentiation of germ cells to mature spermatozoa.

Introduction

The toxic effects of drugs and environmental chemicals on the human reproductive system have become a major health concern; incidences of chemically induced germ cell damage and sterility appear to be on the increase. Recently in the United States, male factory workers occupationally exposed to 1,2-dibromo-3-chloropropane (DBCP) became sterile, evidencing oligospermia, azoospermia, and germinal aplasia. Factory workers in battery plants in Bulgaria, lead mine workers in the state of Missouri, and workers in Sweden who handle organic solvents (toluene, benzene, and xylene) suffer from low sperm counts, abnormal sperm, and varying degrees of infertility. Diethylstilbestrol (DES), borax, cadmium, methylmercury, and many cancer chemotherapeutic agents have been shown to be toxic to the male and female productive system and, thus, possibly capable of also inflicting genetic damage to germ cells (*1–10*).

It has recently been reported that the average or "normal" sperm counts in American men may have dropped over the past 25 years. A study in 1950 which sampled 1000 men and showed 44% with sperm counts of 100 million or more sharply contrasts with a study published in 1977, where only 22% of 2000 men tested had sperm counts of 100 million or more. The percentage of men with sperm counts in the 20 million to 40 million range rose from 12% in the 1950 study to about 22% in 1977 (*11*).

Such findings in themselves warrant more intensive examination of chemically induced infertility and the development of methods to assess potential genetic and toxic effects of environmental chemicals on germ cells. In particular, they warrant the investigation of pharmacokinetic factors within the testis which combat the toxicity of environmental chemicals: the blood-testis barrier (BTB), enzymatic activation and inactivation of chemicals, and DNA repair. These factors are fundamental to our

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understanding of which environmental chemicals and at what active chemical concentrations are likely to reach the germ cell in the testis and irreparably damage its DNA and other macromolecules. Furthermore, these investigations may offer other significant benefits; they may increase our understanding of two major problem areas in toxicology: the extrapolation of laboratory data to man and the establishment of realistic dose levels for testing toxic properties of environmental chemicals and, ultimately, for setting safe levels of exposure.

In our theorizing about chemically induced toxicity, it seems sometimes forgotten that a chemical, to exert toxic effects, must achieve a biologically effective concentration at the site of action; the dose level must be high enough to overwhelm the onsite detoxification and/or biochemical repair systems. With respect to carcinogenesis, overwhelming detoxification processes in an organ is particularly important, because reactive electrophiles will react with DNA and/or other macromolecules, a currently recognized mechanism for carcinogenesis as well as other toxicity. Furthermore, in a partially theoretical examination of dose-response relationships with chemical carcinogens, Gehring (*12*) demonstrated that high test doses of chemicals likely to overwhelm excretion mechanisms, adaptation systems and repair processes did not extrapolate linearly to the more biologically realistic lower doses. He concluded that "currently employed extrapolation techniques are likely to overestimate risk." Applied to the testis, Gehring's findings indicate that toxicity of an environmental chemical is inversely related to the capacity of pharmacokinetic factors to prevent or repair damage to the germ cell and its DNA.

By describing the complexities of the passage of drugs and other toxic chemicals from the blood to the male germ cells in the seminiferous tubules, this paper hopes to elucidate the mechanisms of the pharmacokinetic factors and their capabilities and provide data that is pertinent to the extrapolation of laboratory data to man and the establishment of dose levels.

Anatomy of the Mammalian Testis

Within the scrotum, the testis is covered with a fibrous connective tissue, the tunica albuginea, from which thin partitions, or septa, project into the organ, dividing it into lobules. Each lobule contains many convoluted seminiferous tubules, wherein spermatogenesis—the continuous production of functional male germ cells—occurs.

The vascular anatomy of the testis is comprised of the internal spermatic artery, testicular artery and the associated venous network. The testicular artery enters the testis at the cranial pole and branches into testicular arteries which accompany the tunica albuginea septa, thus, supplying blood to the lobules and all other parts of the testis. Within the lobules, the epithelium of the seminiferous tubules lies against a basement membrane which is surrounded by several layers of thin myoid cells. Between the seminiferous tubules are interstitial compartments consisting of Leydig cells richly bathed in blood and lymph. Capillaries, closely attached to and surrounding the seminiferous tubules, run both parallel and at right angles to the tubules. These capillaries have been demonstrated angiographically (*13*). The veins originating within the interstitial compartment of the testis return tortuously to the cranial pole of the testis, where they drain into the base of the pampiniform plexus (Fig. 1).

There are at least three major fluid compartments in the testis. The first compartment is formed by the blood supplied by the testicular artery, perfusing slow and almost nonpulsatile, through the capillary network in the interstitium, and draining into the pampiniform venous network. Testicular arterial blood flow is shown to be 12.0 ml/g testis/hr in the rat. Secondly, there is the interstitial fluid lying

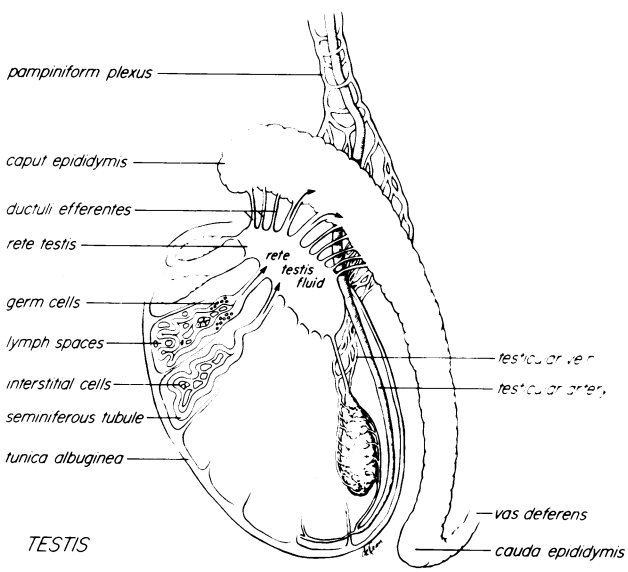

FIGURE 1. Schematic diagram of rat testis. Depicted are the major fluid compartments; arteries and veins connected by intertubular capillaries; interstitial cells lying between the seminiferous tubules and exposed to both lymph and intertubular capillaries. Seminiferous tubule fluid is secreted within the tubules and flows toward the rete testis carrying mature sperm through the ductuli efferentes into the caput epididymis.

between the tubules which drains by way of interstitial lymphatics into lymphatics in the septa and then into larger vessels following the course of the spermatic veins to the inguinal canal. Testicular lymph flow was estimated to be 35 μl/g testis/hr in the rat. Thirdly, fluid formed in the seminiferous tubules and the rete testis flows to the caput epididymis by way of ductuli efferentes carrying spermatozoa formed in the seminiferous tubules. Rete testis fluid is reabsorbed in the caput epididymis. The rate of flow into the rete testis is much slower than that of either testicular blood or lymph and is not related to spermatozoa production. Rete testis fluid has a unique ionic and chemical composition, is closely regulated and thought to be actively secreted by the Sertoli cells. In our laboratory and others, 50-80 μl of rete testis fluid per testis (CD-rat) have been obtained consistently (*14–16*).

When studying the mutagenic and toxic potentials of environmental chemicals on male germ cells, it has been generally assumed that test chemicals readily gain access to the germ cells in the seminiferous tubules, tubuli recti, rete testis, ductuli efferentes, epididymis and vas deferens. Such an assumption, however, ignores many pharmacokinetic concepts regarding the distribution of chemicals in the testis and male accessory gland. Figure 2 presents diagrammatically the pharmacokinetic and biochemical complexities of the male reproductive system: the blood-testis barrier (BTB), enzymatic activation and inactivation of chemicals, interaction with DNA, and DNA repair.

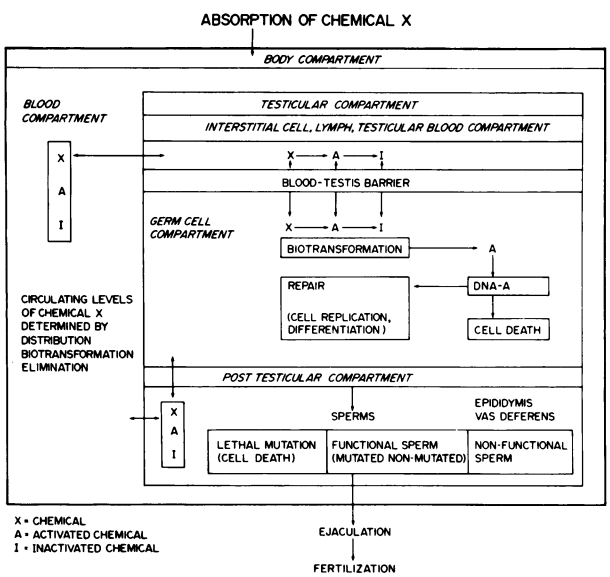

FIGURE 2. Pharmacokinetic model for the male gonad system.

Pharmacokinetic Aspects of the Blood-Testis Barrier

Physiological evidence for the existence of the blood-testis barrier (BTB) was reported by Setchell (*14*). He demonstrated that immunoglobulins and iodinated albumin, inulin, and some small molecules were excluded from the seminiferous tubules by the BTB. Dym and Fawcett (*17*), studying the distribution of low molecular weight protein, horse radish peroxidases, and the lanthanum ion in the testis, suggested that the primary permeability barrier for the seminiferous tubules was the surrounding layers of myoid cells; where these layers were breached, specialized Sertoli cell-to-Sertoli cell junctions within the seminiferous epithelium constituted a secondary Sertoli cell-to-Sertoli cell barrier. However, Dym and Fawcett's use of such large molecules to define the anatomical site of BTB is an inadequate method, for the molecules encountered in the environment can be significantly smaller.

To define the functional BTB, we investigated some of the pharmacokinetic parameters governing the movement of chemicals and drugs across the BTB into the male testis. In these studies, the permeability of nonelectrolytes with varying molecular sizes and of barbiturates, salicylic acid, and sulfonamides with varying partition coefficients and pK_a values was determined.

The movement of the nonelectrolytes indicated that the permeability of compounds across the BTB depends on their molecular size. As expected, it was found that plasma water rapidly attained equilibrium with the rete testis fluid concentration; urea accumulated more slowly, approaching the plasma water concentration by 120 min; galactose moved very slowly across the BTB; and the accumulation of inulin was almost negligible. The molecular weights and transfer rate constants for these nonelectrolytes are presented in Table 1.

Apparently molecules smaller than 3.6 Å (e.g., water, urea) can be transported readily across the BTB, while larger molecules such as galactose and inulin cannot. Transport of these small hydrophilic molecules correlated well with their molecular size

Table 1. Transfer rates of chemicals with various molecular weights into rete testis fluid.

Compound	Molecular weight	Transfer rate K, min^{-1}
THO	20	> 0.2
Urea	60.1	0.030 ± 0.005
Galactose	180.2	0.002 ± 0.001
Inulin	5000	< 0.001

and suggests a pore radius in the range of 2–4 Å, a size much more restrictive than that which exists in muscle capillaries.

Permeability studies of drugs with varying lipid solubilities, such as thiopental, pentobarbital, barbital, sulfamethoxypyridazine, sulfanilamide, sulfaguanidine, and salicylic acid, demonstrated that such drugs do not attain rete testis concentrations greater than plasma and that the transport processes appear to be exponential with time. Transport rate constants derived from $K = (-1/t) \ln (C_p - C_{rtf}/C)$ for all drugs studied plot as straight lines; the rates of entry into the rete testis fluid were proportional to the concentration gradient in accordance with Fick's law (Fig. 3). Transfer rate constants derived from the slopes of the seven test chemicals demonstrated good correlation between their respective lipid solubilities and membrane penetrabilities (Table 2), suggesting that the rate-limiting factor for transport of these compounds across the BTB is the lipid solubility of chemicals at physiological pH.

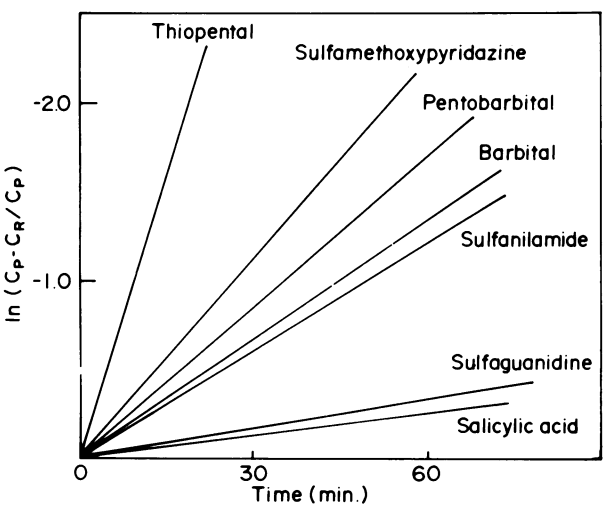

FIGURE 3. Relative rates of transport of various chemicals into the rete testis fluid. The slope of each line is the rate constant. C_R represents chemical concentration in the rete testis fluid; C_p represents unbound concentration of chemicals in the plasma.

However, the permeability characteristics of the BTB are generally similar to those membranes which limit penetration into the central nervous system (*18*), gastrointestinal tract (*19, 20*) and mammary gland (*21*). Moreover, the data are similar to those reported for water, urea, and inulin in the cerebrospinal fluid and aqueous humor (*22*).

According to the generally accepted hypothesis, chemicals penetrate the BTB in their nonionized form since this form is most lipid soluble. A comparison of the transfer rates of the acidic drugs tested shows that the lipid solubility of the nonionized moiety correlates well with transfer rates. Thiopental and barbital provide a striking example of the importance of lipid solubility; the compounds have nearly identical pK_a but are vastly different in their lipid solubilities and, consequently, in their abilities to penetrate biologic membranes.

The transfer rates of sulfonamides and salicylic acid also correlate well with lipid solubility. Transfer rate constants of these drugs are generally very close to the values reported for penetration to the cerebrospinal fluid (*23*). On the other hand, while the transfer constant for barbital is similar to that reported for the blood-brain barrier, the values determined for thiopental and pentobarbital in the BTB are lower than reported for the BBB (*24*).

Although transport of salicylic acid across the intestine (*25*) and the kidney cortex (*26*) is reportedly an active process, these experiments did not suggest such a process for the BTB. Salicylic acid enters the rete testis fluid slowly because of its high degree of ionization at pH 7.4. None of the compounds in the present study appeared to be transported actively. However, certain antibiotics (*27*), *p*-aminohippuric acid (*28*) and some metal ions such as zinc and cadmium (*29*) appear to reach concentrations in the rete testis fluid that are greater than free levels in the plasma.

These data indicate that the major transport process of chemicals across the BTB depends on their lipid solubility and molecular size. Transfer rate

Table 2. Relation of the transfer rates to physicochemical characteristics.

Drug	Molecular weight	pK_a	Partition coefficient[a]	Transfer rate K, min $^{-1}$ [b]
Thiopental	242.3	7.6	102	0.106 ± 0.010
Pentobarbital	226.3	8.1	21	0.028 ± 0.002
Barbital	184.2	7.5	2.0	0.022 ± 0.006
Sulfamethoxypyridazine	280.3	6.7	1.6	0.037 ± 0.012
Sulfanilamide	172.2	10.4	0.027	0.020 ± 0.003
Sulfaguanidine	232.2	12.1	0.0018	0.0053 ± 0.0009
Salicylic acid	138.1	3.0	0.0003	0.0041 ± 0.0009

[a] Partition coefficients were measured between $CHCl_3$ and pH 7.4 phosphate buffer.
[b] Rate constant for the transfer of drugs from plasma to rete testis fluid.

constants of all drugs tested correlated well with partition coefficients and, in general, penetration obeyed simple diffusion kinetics. Thus, the rates of penetration of acidic drugs into seminiferous tubules can probably be predicted on the basis of partition coefficients.

In summary, the BTB is apparently a complex multicellular system composed of both myoid cells surrounding the seminiferous tubules and several highly organized layers of spermatogenic cells within the tubules. This system restricts the permeability of many foreign compounds to male germ cells and, thus, must be factored into the pharmacokinetic model when investigating the reproductive and mutagenic effects of environmental chemicals, and extrapolating these data to man.

Activation and Inactivation of Environmental Chemicals in Testis

It has been shown previously that numerous direct alkylating agents interact and bind with germ cell DNA (*30*) without requiring metabolic activation. Other classes of environmental chemicals, however, require metabolic activation in order to exert their toxicity. For example, it is assumed that polycyclic hydrocarbons, hydrazines, DBCP, and cytoxan require metabolic activation by the hepatic mixed function oxidases in order to affect toxic actions on target organs. Dimethylbenzanthracene produces reversible effects in rats by affecting both spermatogonia and spermatocytes (*31, 32*). Treatment of male mice with benzo[a]pyrene produces a significant increase in early embryonic death, suggesting a dominant-lethal mutation of germ cells (*33*). Rats treated with 3-methylcholanthrene show significant decrease in testicular cytochrome P-450 and a concomitant decrease of testosterone levels (*34*).

What about the ability of testicular tissue to activate nonreactive chemicals? In tissues other than germ cells, the toxic effects of polycyclic hydrocarbon have been demonstrated to be due to its active metabolite, an epoxide, which interacts with DNA, RNA and other macromolecules. A steady-state level of epoxide(s) within the cells of a target organ is obviously a function of the metabolite's rate of formation and degradation and the sensitivity of the organ to such toxic metabolite(s). Consequently, the rates of epoxide forming and detoxifying enzyme activities in various tissues or cells may be an important determinant of tissue specific toxicity. Thus, we have determined the differential distribution of aryl hydrocarbon hydroxylase (AHH), epoxide hydrase (EH), glutathione *S*-transferase (GSH-ST) and cytochrome P-450 content in interstitial and spermatogenic cells in the seminiferous tubules.

The relative specific activities of AHH, EH, GSH-ST and cytochrome P-450 content in adult testes and liver are shown in Table 3. Appreciable activities of both mixed-function oxidases and epoxide-degrading enzymes, as well as cytochrome P-450 were found in testicular tissue. Glutathione *S*-transferase activity was especially high. The distribution of these enzymes and cytochrome P-450 in the interstitial and germ cell compartment indicate that AHH activity and cytochrome P-450 content of microsomes from the interstitial cells were nearly two-fold greater than that in the tubules (Table 4). In contrast, the specific activities of the detoxification enzymes, EH and GSH-ST, in tubules were twice that in the interstitial cells. Although AHH activity in interstitial cell microsomes was only 5.0% that of hepatic microsomes, its close proximity to the germ cells may be important for enzyme activated toxicity.

Therefore, factors affecting induction of AHH and cytochrome P-450 probably play a significant role in germ cell toxicity. It has been demonstrated

Table 3. Epoxide hydrase, glutathione *S*-transferase, and aryl hydrocarbon hydroxylase activities and cytochrome P-450 content of microsomes or 176,000*g* supernatant fraction from adult rat testis.

Enzyme	Specific activity or content, mean ± SD (*n*)	
	Testis	Liver
Glutathione *S*-transferase, nmole product/min-mg protein with benzo[a]pyrene 4,5 oxide substrate[a]	19.99 ± 1.11 (8)	41.29 ± 2.10 (4)
Epoxide hydrase, nmole product/min-mg protein with benzo[a]pyrene 4,5-oxide substrate[b]	0.77 ± 0.06 (8)	10.85 ± 1.68 (4)
Aryl hydrocarbon hydroxylase, pmole 3-hydroxybenzo[a]pyrene formed/min-mg protein[b]	5.17 ± 0.58 (4)	106 ± 8.3 (6)
Cytochrome P-450, nmole/mg protein[b]	0.125 ± 0.018 (4)	0.85 ± 0.03 (6)

[a] 176,000*g* supernatant.
[b] Microsomes.

Table 4. Epoxide hydrase, glutathione *S*-transferase, and aryl hydrocarbon hydroxylase activities and cytochrome P-450 content of microsomes or 176,000*g* supernatant fraction prepared from rat testicular interstitial and spermatogenic cells.

Enzyme	Specific activity or content	
	Interstitial cells	Spermatogenic cells
Glutathione *S*-transferase, nmole/min protein[a]	65.3 ± 4.8 (3)	119 ± 6.8 (3)
Epoxide hydrase, nmole/min-mg protein[b]	1.09 ± 0.32(3)	2.36 ± 0.52 (3)
Aryl hydrocarbon hydroxylase, pmole/min-mg protein[b]	5.98 ± 0.58 (3)	3.18 ± 0.32 (3)
Cytochrome P-450 content, nmole/mg protein[b]	0.196 (2)	0.084 (2)

[a] 176,000*g* supernatant.
[b] Microsomes.

that enzyme induction by phenobarbital significantly enhanced 2-acetylaminofluorene (AAF)-induced hepatocarcinoma in mice, which suggests that the induction of activating enzymes may enhance the expression of tumor by naturally occurring carcinogens.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo[a]pyrene significantly induce both testicular and prostatic AHH activity and cytochrome P-448. AHH activity induced by TCDD in rat testis and prostate gland was 2 and 150 times that of its controls, respectively (Figs. 4 and 5). Thus, exposure to environmental chemicals can induce significant levels of activating enzyme systems in male testis as well as in the prostate gland, which suggests further modulation of genetic toxicity of germ cells as well as potential tumorigenicity of prostate glands.

DNA Repair in Germ Cell Toxicity

The discovery that bacteria subjected to lethal doses of ultraviolet light partially recovered their ability to form colonies if subsequently exposed to strong visible light provided the first evidence that cells might contain repair enzymes for radiation-damaged DNA (*35*). This observation encouraged the application of biochemical methods to elucidate the DNA repair mechanism. There are many indications that the mammalian cell recognizes and removes DNA damaged as a consequence of treatment with a variety of chemical agents. Moreover, there appear to be two distinct mechanisms by which this repair of mammalian cell DNA can be achieved. The first is referred to as excision repair or "cut and patch" repair; the chemical region in the DNA is recognized by a complex enzyme system that excises the modified base from the DNA,

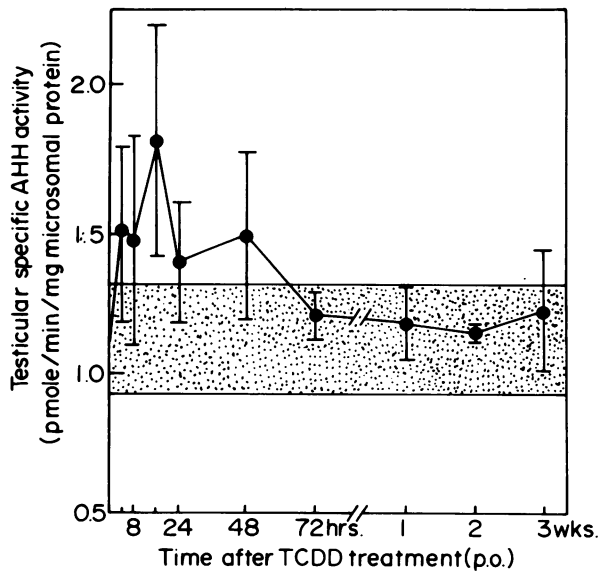

FIGURE 4. Time course of testicular AHH enzyme activity after a single PO treatment of TCDD (10 μg/kg body weight). Control and experimental values are represented by the shaded range and solid circles, respectively. Each point with a bar represents mean ± SD (*n* = 3).

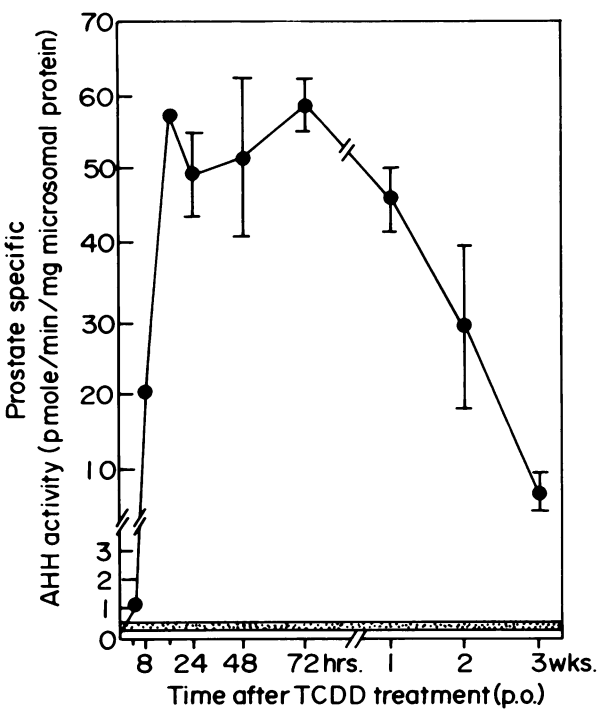

FIGURE 5. Time course of prostatic AHH enzyme activity after a single PO treatment of TCDD (10 μg/kg body weight). Control and experimental values are represented by the shaded range and the solid circles, respectively. Each point with a bar represents mean ± SD (*n* = 3).

degrades it, resynthesizes a section to reconstitute the original DNA strand, and then inserts this into the preexisting DNA. Evidence for this form of repair has come from measurements of the loss of mutagen-induced substituents on DNA (*36*) and from the detection of nonsemiconservative DNA synthesis, sometimes called "repair synthesis" in alkylated cells (*37*). The uptake of ^{3}H-thymidine into cells that are not in the S-phase of the cell cycle is generally indicative of unscheduled DNA synthesis (*38*).

The second type of repair in mammalian cells is the so-called post-replication DNA repair (*39*). It was found that DNA strands synthesized in irradiated mammalian cells during a thymidine labeling period of 30–60 min are of lower than normal molecular weight. This suggests that they were synthesized only along the undamaged portions of the DNA template. With further incubation, these low molecular weight nascent chains become joined into strands of original molecular weight following cell division. The pyrimidine dimers remain in the original DNA templates. Therefore, this type of DNA repair mechanism which bypasses damage in the DNA templates is thought to be another important mechanism for mammalian cell DNA repair.

So far, it has been shown that both physical (ultraviolet and x-ray radiation) and chemical carcinogens can cause damage to DNA molecules. Damage inflicted on the DNA templates, unless repaired, may interfere with transcription or replication and result in lethal mutations (cell death), mutations which develop into transformed cells or genetic mutations which can be amplified in the case of germ cells by generations of genetic recombinations. Genetic deficiencies in the DNA repair system are found among people with the disease called xeroderma pigmentosum, a skin cancer induced by sunlight.

In an attempt to better understand the mechanism of action and also detect germ cell damage induced by chemicals, DNA repair activity in various spermatogenic cell types has been investigated. A combination of several biochemical techniques, including velocity sedimentation cell separation (*40*), alkaline gradient centrifugation (*41*) and alkaline elution analysis (*42*) was applied to spermatogenic cells.

Dominant lethality of alkylsulfonates has been widely studied in the past. The results indicated that a homologous series of alkylsulfonates affect different stages of spermatogenic cells. For example, methylmethane sulfonate (MMS) (monofunctional alkylating agent) affects only spermatozoa and spermatids and no other spermatogenic cells (Fig. 6). Various explanations of these differential effects

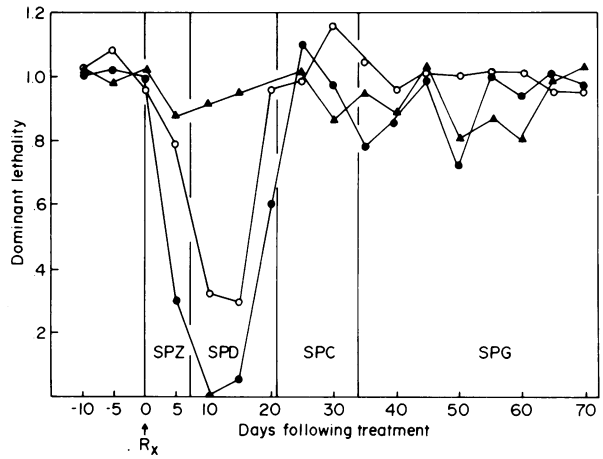

FIGURE 6. Dominant lethality after a single intraperitoneal administration of MMS at (▲) 22.5, (○) 45 and (●) 90 mg/kg body weight of male CD_2F_1 mice. Dominant lethality is expressed as total live embryo of experimental animals/total live embryo of the controls. Virgin female CD_2F_1 were allowed to cohabitate with either control or treated male mice for 5 days. Each point represents 10 animals.

of alkylsulfonates have been proposed. Existence of a blood-testis barrier which could effectively exclude MMS from the seminiferous tubules while allowing penetration into the epididymis and vas deferens is one possibility. Other workers suggest a selective uptake of chemical by the various germ cell types; however, autoradiographic studies with tritium-labeled MMS offer no evidence for selective localization in any spermatogenic cell types. Since MMS should be capable of alkylating nucleophilic sites irrespective of cell types, the selective toxic effects on spermatids and spermatozoa might be attributed to differences in the expression of toxicity by alkylated cells. For example, DNA repair deficiency could result in the selective toxicity of spermatozoa and spermatids.

To study unscheduled DNA synthesis after MMS treatment, male mice were first treated with varying doses of MMS (16.3, 32.5, and 65 mg/kg) for 2 hr, followed by the intratesticular administration of ^{3}H-thymidine 1 hr before sacrifice. Spermatogenic cell types were isolated by the velocity sedimentation cell separation technique (*40*). Seminiferous tubules were removed from animals, minced, and digested with collagenase (0.1%) at 32°C for 30 min. The cell suspension was then filtered and the cells counted. Twenty million cells were loaded into the sedimentation chamber and a 1 to 3% Spinner-albumin gradient was developed. A period of 4 hr was allowed for cells to sediment before fractionation.

The control mice receiving saline followed by intratesticular injection of ^{3}H-thymidine showed a

single peak of radioactivity which is associated with spermatogonial cells passing through S-phase (Fig. 7). In contrast, the radioactive profiles obtained after MMS treatment demonstrated that thymidine is incorporated not only into spermatogonia but also into the leptotene, zygotene, pachytene, and diplotene cells in decreasing order. The extent of unscheduled DNA synthesis was inversely related to dose. Normally no DNA synthesis occurs in these premeiotic cells; therefore, the thymidine incorporation in untreated cells is very low.

Radioactivity in spermatogonial cell DNA after MMS treatment is also 1.5 to 2.0 times greater than that of control. This finding suggests that non-S-phase spermatogonial cells were also induced to undergo unscheduled DNA synthesis. In contrast, no radioactivity was associated with spermatids or spermatozoa (spermiogenic cells). Therefore, spermiogenic cells appear unable to repair DNA damage and thus are vulnerable to the effects of the monofunctional alkylating agent, MMS.

A modified alkaline sucrose density gradient technique was used to determine the dose-response relationship for MMS-induced DNA single strand breaks and the time-response for subsequent repair. Prepubertal mice were used to obtain relatively pure fractions of spermatocytes and spermatogonia, and the alkaline sucrose density gradient technique was compared to the alkaline elution technique.

Male mice were treated intraperitoneally with 0.5 μCi of tritium labeled thymidine per gram of body weight three times daily for 5 days prior to MMS treatment. Figure 8 demonstrates the dose-response relationship following MMS doses of 22.5, 45, and 90 mg/kg. The mice were killed 1 hr after MMS treatment, and the spermatogenic cells gently layered on the alkaline detergent surface of the gradient. After 4 hr, the gradient was centrifuged at 15,000 rpm for 16 hr at 4°C. All steps of the alkaline sucrose density gradient procedure were carried out in the dark. These results indicate that MMS-induced DNA breaks are dose related, and premeiotic spermatogenic cells are capable of repair.

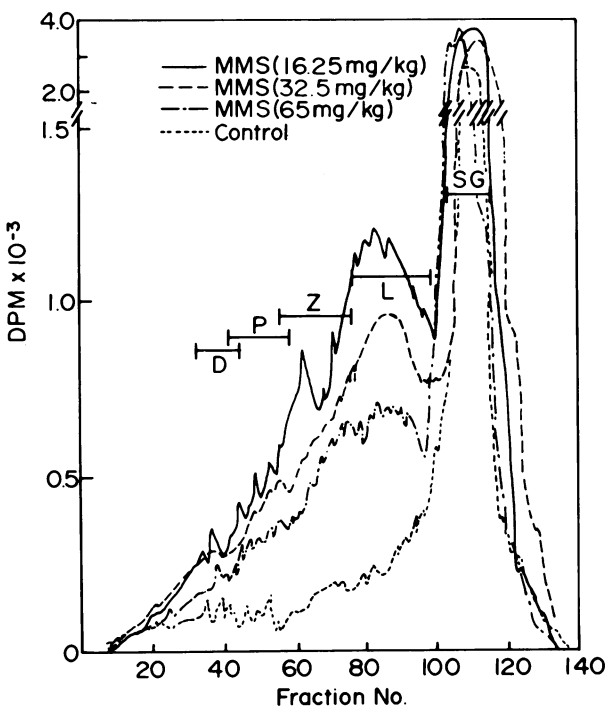

FIGURE 7. Control and MMS-induced changes in the radioactive profiles of various spermatogenic cells after velocity sedimentation cell separation.

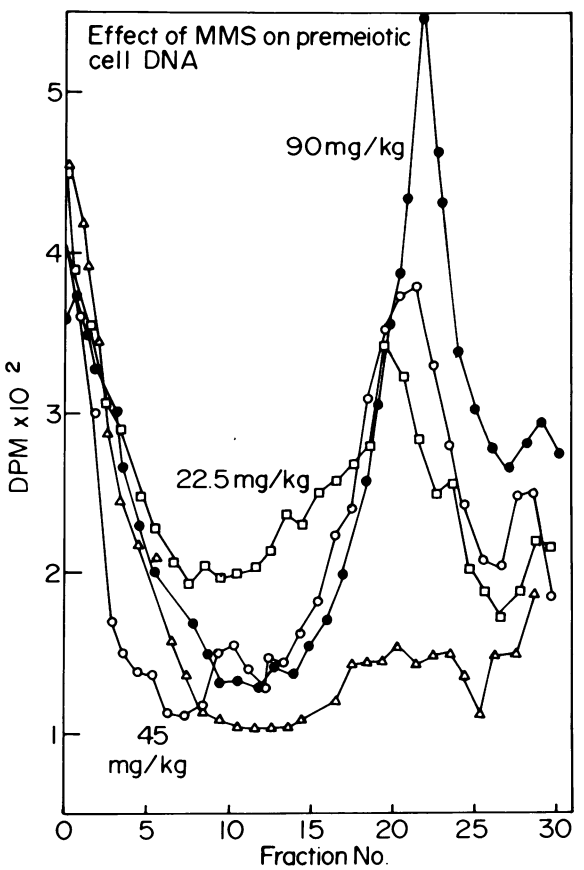

FIGURE 8. Profiles of DNA single-strand breaks in the alkaline sucrose density gradient at various MMS doses: (□) 22.5 mg/kg; (○) 45 mg/kg; (●) 90 mg/kg; (Δ) saline controls. The gradient is from left to right. Radioactive peaks on the left represent the native DNA and the radioactive peaks on the fractions between the 18 and 25 represent single DNA strands.

The time-response or the rate of DNA repair was estimated by treating prepubertal male mice intraperitoneally with 0.5 μCi of tritium-labeled thymidine per gram of body weight three times daily for 5 days prior to MMS treatment (90 mg/kg). Mice were killed and DNA fractionated at various times following MMS. Single strand DNA breaks induced by a single dose of 90 mg/kg MMS gradually disappeared as a function of time (Fig. 9). After 65 hr, no single-strand DNA breaks could be detected. With doses of 22.5 and 45 mg/kg MMS, the time for complete repair was 8 and 12 hr, respectively. These results indicate that MMS-induced single strand breaks are dose dependent and provide an approximation of the DNA repair rate.

We attempted to compare the relatively more simple alkaline elution technique to the alkaline sucrose density gradient approach. Prepubertal mice were pretreated with tritium-labeled thymidine as previously described. Single-strand breaks, as well as the duration of DNA repair, were subsequently determined. The fraction of total tritium-labeled DNA single strands eluted after 11.25, 22.5, 45, and 90 mg/kg of MMS were 23, 42, 83, and 90%, respectively (Fig. 10). Only single strand breaks are eluted with this technique; native DNA remains on the filter. The durations for complete DNA repair after a single treatment of MMS at 22.5, 45, and 90 mg/kg were found to be 21, 46, 70 hr, respectively (Fig. 11). The results obtained with the alkaline elution technique were similar to those obtained with the alkaline sucrose density gradient technique. The alkaline elution analysis is relatively more convenient and less time consuming than the alkaline sucrose density gradient centrifugation technique.

To further test whether mature spermatozoa have DNA repair capability, a group of male mice were treated intraperitoneally with 0.5 μCi of tritium labeled thymidine three times daily for 5 days. A period of 35 days was allowed for the labeled spermatogonia to differentiate into mature spermatozoa. Peak radioactivity was associated with sperm in the vas deferens 35 days after the first day of radiolabeled thymidine injection. Animals were then treated with 90 mg/kg MMS and spermatozoa collected from the vas deferens. Spermatozoa (10^5 cells) were washed with spinner culture medium, treated subsequently with dithiothreitol to unpack the nucleoproteins, and the DNA subjected to alkaline elution analysis. Results indicated that under

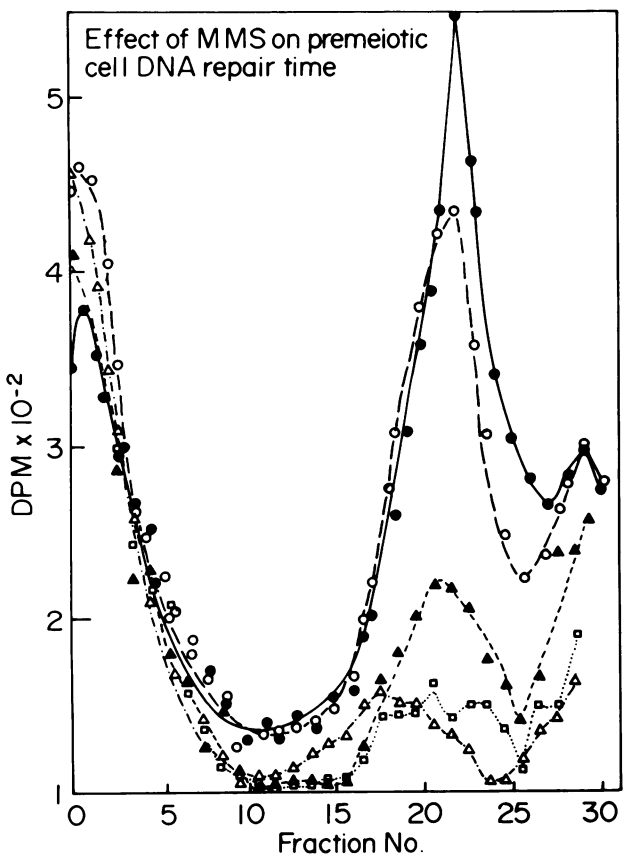

FIGURE 9. Profile of DNA single strands after single administration of MMS: (●) 1 hr; (○) 16 hr; (▲) 48 hr; (Δ) 65 hr; (□) controls. The gradient is from left to right. Radioactive peaks on left represent the native DNA and radioactive peaks on the right represent single DNA strands.

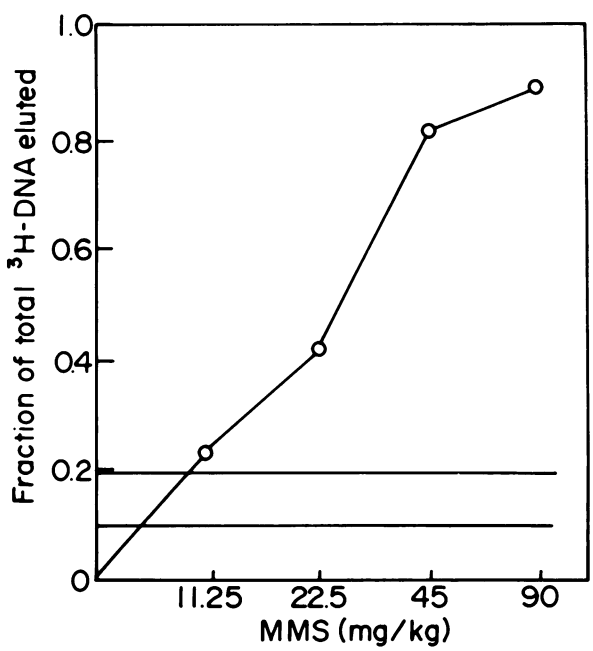

FIGURE 10. Alkaline elution analysis of ^{3}H-DNA. The fraction of total ^{3}H-DNA eluted is dose dependent. (=) represents the range of controls.

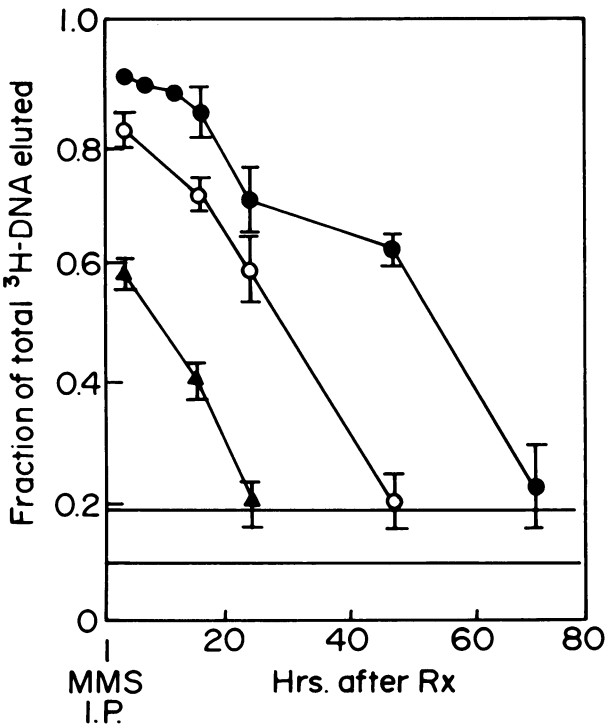

FIGURE 11. Fraction of total ³H-DNA eluted a various times after a single treatment of MMS at (▲) 22.5 mg/kg; (○) 45 mg/kg; (●) 90 mg/kg; (=) represents the range of controls.

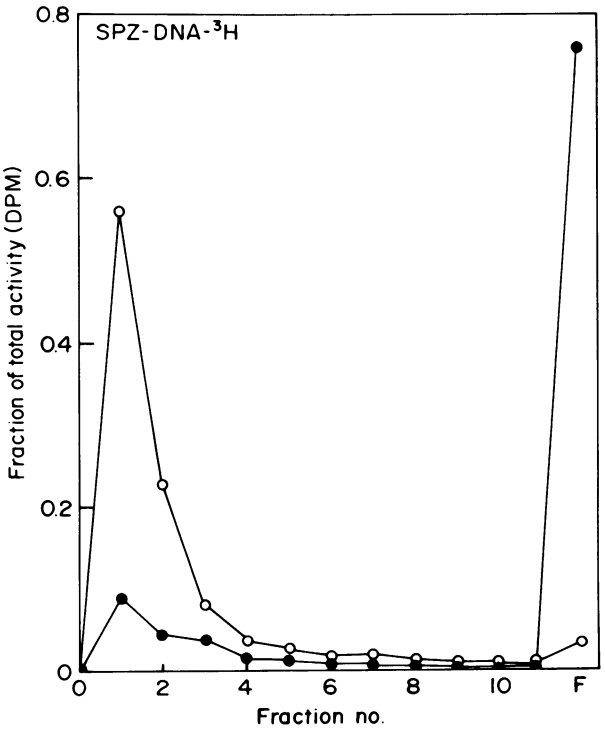

FIGURE 12. Fraction of total ³H-DNA of spermatozoa eluted after (○) a single treatment of MMS at 90 mg/kg; control (●). F represents the fraction of total ³H-DNA remaining in the filter (PVC Millipore filter, 2 μ pore size).

these conditions single strand breaks could be demonstrated in spermatozoa following MMS treatment (Fig. 12).

These studies suggest that the possible mechanism for the lack of dominant lethal effects of spermatogonia and spermatocytes (premeiotic spermatogenic cells) is due to their ability to repair DNA while spermatids and spermatozoa (spermiogenic cells) are unable to repair DNA and, thus, lethal mutations are expressed. The lack of DNA repair in the mature cell types might be attributed to an absence of any of the DNA repair enzymes; this hypothesis requires further study.

DNA single-strand breaks induced by MMS in premeiotic spermatogenic cells appear to be dose dependent. The DNA repair systems can probably be saturated at higher doses of MMS. Overwhelming the repair system would result in a larger number of affected cells. Thus, the DNA repair system is another protective mechanism with regard to toxic effects of environmental chemicals and drugs. Repair rates need to be quantitated and factored into the pharmacokinetic model described. The DNA damage measured by the alkaline elution analysis appears to be a relatively simple, sensitive indicator of chemical injury in germ cells.

In conclusion, understanding the pharmacokinetic characteristics of the BTB, toxification and detoxification systems as well as DNA repair systems in male gonads will allow a better understanding of the reproductive and genetic toxic effects, and increase the reliability of extrapolation in data from laboratory animals to man and the resulting estimates of risk.

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